Viral capture

strain overexpressing *patS* (*10*).

We propose a model in which a processed PatS peptide, originating from differentiating proheterocysts, diffuses along the filament's contiguous periplasmic space and is taken up by neighboring cells, creating a gradient of inhibitory signal. The intracellular target of PatS signaling is unknown, but components of a phosphorelay such as that found in *Bacillus* (*13*) are likely candidates.

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7. pAM1697 contains *patS* that was amplified by PCR and cloned into pPet1 (W. J. Bulkema and R. Haselkorn, personal communication), which contains the *petE* promoter. pAM1714 contains the P_{petE}-*patS* fragment from pAM1697 in pAM504 (*19*). pAM1715 contains *patS* in the reverse orientation. Two independent clones for each construct were tested, and they produced similar results.
8. pAM1882 DNA (1 μg) was incubated in a final volume of 100 μl of 0.4 M hydroxylamine-HCl in buffer [50 mM sodium phosphate (pH 6.0) and 0.9 mM EDTA] at 65°C for 60 min [H. C. Lee, Y. P. Toung, Y. S. Tu, C. P. Tu, *J. Biol. Chem.* 270, 99 (1995)]. After dialyzing the DNA against TE buffer [10 mM tris-HCl (pH 8.0) and 1 mM EDTA] overnight, 2 μl was used to transform *Escherichia coli* conjugal donor strain AM1359 (strain DH10B containing pRL623 and pRL443) [J. Elhai, A. Vepritskiy, A. M. Muro-Pastor, E. Flores, C. P. Wolk, *J. Bacteriol.* 179, 1998 (1997)]. Several thousand transformant colonies were collected and used for conjugation with *Anabaena* PCC 7120. After incubating for 10 days on BG-11_0 plates containing neomycin (25 μg/ml), the four best-growing Het^+ exconjugants were selected for plasmid isolation and DNA sequencing.
9. In-frame (pAM1899) and out-of-frame (pAM1860) *patS-lacZ* translational fusions were made by ligating *patS* fragments to *lacZ*. *Anabaena* PCC 7120 exconjugants AMC446 (pAM1899) and AMC448 (pAM1860) were induced in BG-11_0 for 0, 6, 14, 18, 27, and 48 hours and harvested for β-Gal assays. Cells were lysed and assayed as previously described [M. R. Schaefer and S. S. Golden, *J. Bacteriol.* 171, 3973 (1989)], except that the filaments were frozen at −85°C before processing. β-Gal was measured and expressed as specific activity (nanomoles of *o*-nitrophenyl-β-D-galactopyranoside per minute per milligram of protein).
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15. AMC451 was made by double recombination with suicide plasmid pAM1702 (Fig. 2A) as previously described (*19*). pAM1702 contains *patS*-flanking sequences and an Ω Sp′/Sm′ cassette [conferring spectinomycin and streptomycin resistance] in *sacB*-containing suicide vector pRL278 [T. A. Black, Y. Cai, C. P. Wolk, *Mol. Microbiol.* 9, 77 (1993)]. The Ω Sp′/Sm′ cassette replaces a 381-bp Eco RV–Sca I fragment containing the entire *patS* gene. Four independent isolates with identical phenotypes were obtained after selection on media containing sucrose (5%), spectinomycin (2 μg/ml), and streptomycin (2 μg/ml). The structure of the deletion was confirmed by Southern (DNA) blot analysis.
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26. We thank L. Whorff for contributions to the initial analysis of cosmid 8E11; A. Ott for technical assistance; and W. J. Bulkema, J. Elhai, S. S. Golden, M. D. Manson, and members of our laboratory for critically reading the manuscript. Supported in part by NIH grant GM36890.

23 July 1998; accepted 29 September 1998

Binding of Hepatitis C Virus to CD81

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Chronic hepatitis C virus (HCV) infection occurs in about 3 percent of the world's population and is a major cause of liver disease. HCV infection is also associated with cryoglobulinemia, a B lymphocyte proliferative disorder. Virus tropism is controversial, and the mechanisms of cell entry remain unknown. The HCV envelope protein E2 binds human CD81, a tetraspanin expressed on various cell types including hepatocytes and B lymphocytes. Binding of E2 was mapped to the major extracellular loop of CD81. Recombinant molecules containing this loop bound HCV and antibodies that neutralize HCV infection in vivo inhibited virus binding to CD81 in vitro.

HCV is a positive strand RNA virus of the flaviviridae family (*1*) chronically infecting about 170 million persons worldwide (*2*). Chronic HCV infection results in liver diseases (hepatitis, cirrhosis, and hepatocellular carcinoma) in a sizable fraction of cases (*3*). Infection with HCV is also associated with most cases of type II and type III cryoglobulinemia, B lymphocyte proliferative disorders characterized by polyclonal B cell activation and autoantibody production (*4*). The complete HCV sequence has been available since 1989 (*5*); however, progress in understanding the viral life cycle has been hampered by the lack of virus culture systems in vitro. Although hepatocytes and B lymphocytes are thought to be infected by HCV (*1*), there is no consensus on viral tropism, and the cellular receptor for the virus has not been identified.

We have shown previously that a recombinant form of the major envelope protein (E2) of HCV binds with high affinity to human lymphoma and hepatocarcinoma cell lines, whereas it does not bind to mouse cells (*6*). Furthermore, in chimpanzees vaccinated with recombinant E1 and E2 envelope proteins, protection from homologous HCV challenge correlated with the presence of antibodies capable of inhibiting the binding of E2 to human cells (*6*). These results suggested that E2 may be responsible for binding of HCV to target cells.

To identify the E2-binding molecule on human cells, we prepared a cDNA expression

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Exhibit B

library from A2R cells, a subclone of the human T cell lymphoma Molt-4, which exhibited high E2-binding capacity (*7*). This library was screened by transient transfection of a mouse fibroblast cell line (WOP) (*8*) with E2 as a probe (*9*). This approach resulted in the identification of a cDNA clone that conferred E2-binding capacity to WOP cells upon transient transfection. This clone contains an insert of 0.9 kb encoding human CD81. This widely expressed 25-kD molecule is a member of the tetraspanin superfamily (*10*), which includes cell surface proteins that span the membrane four times, forming two extracellular loops. The intracellular and transmembrane domains of CD81 are highly conserved among different species, whereas the major extracellular loop is quite diverse (Fig. 1). The major loop is highly conserved in humans and chimpanzees, which are the only known species permissive to HCV infection (*1*) and whose cells bind HCV E2 (*6*).

To confirm that CD81 was the human cell surface molecule binding HCV E2, we used recombinant E2 and antibodies to CD81 (anti-CD81) to assess this interaction. Recombinant E2 competitively inhibited the binding of anti-CD81 to Epstein-Barr virus (EBV)–transformed B cell lines (EBV-B cells) (Fig. 2A). In addition, E2 reacted in protein immunoblots with anti-CD81–precipitated material (Fig. 2B). CD81 on fresh lymphocytes and hepatocytes is also capable of binding E2, as demonstrated by immunohistochemical staining with biotin-labeled E2 (*11*).

Because of the lack of HCV culture assays in vitro, we developed alternative methods to demonstrate that CD81 interacts with HCV. We prepared a recombinant fusion protein (Fig. 3A) expressing the major extracellular loop (EC2) (amino acid residues 113 to 201) of human or mouse CD81 fused to the COOH-terminal end of thioredoxin (TRX-EC2) (*12*). The proteins containing the human, but not the mouse, loop bound to E2 in protein immunoblot (*13*) and in solution as shown by inhibition of binding of E2 to human cells (Fig. 3B). To assess virus binding, we attached human or mouse TRX-EC2 proteins to polystyrene beads and incubated them with an infectious plasma containing known amounts of viral RNA molecules. After washing, the amount of bead-associated virus was measured by quantitative reverse transcription polymerase chain reaction (RT-PCR) (*14*). Beads coated with human TRX-EC2, but not with mouse TRX-EC2, bound HCV in a concentration-dependent fashion (*15*) (Fig. 3C). Preincubation of beads with anti-CD81 inhibited virus binding. Furthermore, preincubation of the infectious plasma with sera from chimpanzees that were protected from homologous HCV challenge by vaccination with E1 and E2 envelope proteins (*16*) inhibited HCV binding to CD81 (Fig. 3D). In contrast, sera from vaccinated but nonprotected chimpanzees, although containing anti-E2, were not inhibitory (Fig. 3D). These results demonstrate that anti-E2 antibodies, which are capable of neutralizing HCV infection in vivo, can inhibit the binding of HCV to CD81 in vitro, supporting the idea that CD81-E2 interaction is relevant to infection.

Our data demonstrate that human CD81 is sufficient for binding not only E2 but also HCV particles. Given the wide distribution of CD81 (*10*), these results imply that HCV can bind to a variety of cells other than hepatocytes. Consistent with this finding, HCV RNA has been found in T and B lymphocytes and monocytes (*17*). Whether virus binding to CD81 is followed by entry and infection in all cell types is not clear, because it is possible that additional factors are required for HCV fusion or infectivity.

CD81 participates in different molecular complexes on different cell types, a fact that may affect its capacity to mediate HCV attachment or to deliver signals to target cells. For instance, on epithelial and hematopoietic cells, CD81 associates with integrins (*18*), whereas

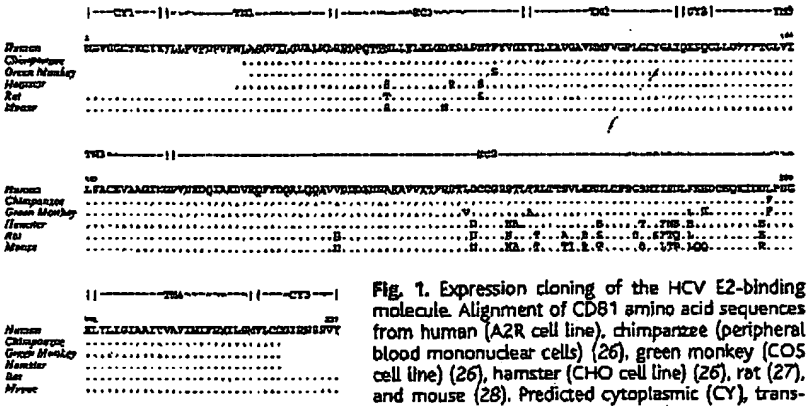

Fig. 1. Expression cloning of the HCV E2-binding molecule. Alignment of CD81 amino acid sequences from human (A2R cell line), chimpanzee (peripheral blood mononuclear cells) (*26*), green monkey (COS cell line) (*26*), hamster (CHO cell line) (*26*), rat (*27*), and mouse (*28*). Predicted cytoplasmic (CY), transmembrane (TM), and extracellular (EC) domains are indicated according to (*10*). Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

Fig. 2. Interaction between recombinant E2 and CD81. (A) Dose-dependent inhibition of anti-CD81 binding to B cells by recombinant E2. EBV-B cells were incubated with increasing concentrations of recombinant E2 for 1 hour at 4°C and then stained with an mAb to CD81 (clone JS-81, Pharmingen). The data are expressed as percentage of inhibition of mean fluorescence intensity (*6*). Preincubation with E2 did not inhibit binding of an anti–major histocompatibility complex class I (clone W6/32) (*29*). (B) E2 detects, on protein immunoblot, the 25-kD protein immunoprecipitated by anti-CD81. A membrane protein extract (about 300 μg) prepared from the A2R cell line was solubilized with 8 mM 3-[[3-cholamidopropyl]-dimethyl-ammonio]-1-propanesulfonate in PBS (pH 7.4) and incubated with 10 μg of recombinant E2 (lanes 2 and 3), 20 μg of anti-CD81 (lane 4), or 20 μg of a control antibody (anti–human CD9; ATCC) (lane 5). After incubation for 2 hours at 4°C, the samples were immunoprecipitated with chimpanzee antisera to E2 (lane 2), chimpanzee preimmune sera (lane 3), or goat anti–mouse IgG (lanes 4 and 5) bound to protein A–Sepharose (CL-4B; Pharmacia). The precipitated samples were eluted in Laemmli buffer, separated by SDS-PAGE under nonreducing conditions, and transferred to a PVDF (polyvinylidene difluoride) membrane by electroblotting. The blots were then probed with recombinant E2 (1 μg/ml) overnight followed by a 2-hour incubation with an mAb to E2 (mAb 291). Visualization of the immunoprecipitated proteins detected by E2 was performed with a peroxidase-conjugated polyclonal anti–mouse IgG (Amersham). As a positive control, a portion of the total membrane extract was also loaded on the gel (lane 1). The mobilities of molecular mass (in kilodaltons) markers are indicated at left. The 25-kD CD81 is immunoprecipitated directly by anti-CD81 or indirectly by a combination of E2 and anti-E2 as shown in lanes 4 and 2, respectively. The high molecular mass bands observed in all lanes (including negative control lane) probably represent cross-reaction of the secondary antibody, detection of E2 precipitated by chimpanzee antisera to E2 subsequently recognized by anti-E2 mAb (lane 2), or detection of mAb to CD81 (lane 4) or mAb to CD9 (lane 5) by the secondary anti–mouse.

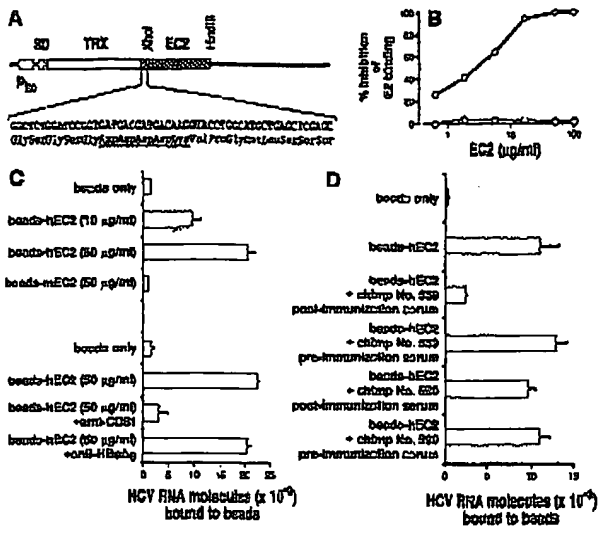

Fig. 3. The major extracellular loop of CD81 binds recombinant E2 and viral particles. (A) Schematic representation of the pThioHis-EC2 plasmid in the region encoding the thioredoxin-EC2 fusion protein. The nucleotide and the amino acid sequences of the joining region are indicated. The enterokinase cleavage site is underlined. P_{tac}, lac promoter; SD, ribosome-binding site; TRX, thioredoxin; EC2, human major extracellular loop (amino acid residues 113 to 201) of CD81. (B) Dose-dependent inhibition of E2 binding to hepatocarcinoma cells by recombinant molecules expressing the EC2 of human CD81. Cells were incubated with E2 and increasing concentrations of human (closed circles) or mouse (open circles) TRX-EC2 for 1 hour at 4°C and then stained with mAb to E2. Data are expressed as percentage of inhibition of mean fluorescence intensity. (C) Binding of HCV to CD81. Polystyrene beads were coated with human (hEC2) or mouse (mEC2) TRX-EC2 (10 or 50 μg/ml) overnight at room temperature (*14*). Each bead was then incubated at 37°C with chimpanzee infectious plasma (genotype 1a) containing 5×10^5 HCV RNA molecules in 200 μl. The bound virus was eluted with lysis buffer, and HCV RNA was measured by quantitative RT-PCR (*14*). Similar results were obtained with an infectious plasma containing HCV of the genotype 1b. For inhibition experiments, the TRX-EC 2–coated beads were incubated with mAb to CD81 or with an isotype-matched irrelevant antibody [hepatitis B surface antigen (anti-HBsAg)] as control, for 1 hour at room temperature before incubation with the virus (*14*). (D) Antibodies that neutralize HCV infection in vivo inhibit binding of HCV to CD81 in vitro. The chimpanzee infectious plasma (200 μl) used in the experiment described in (C) was preincubated at 4°C for 1 hour with 2 μl of serum either from a chimpanzee (number 559) protected from homologous HCV challenge by vaccination with E1 and E2 envelope proteins or from a chimpanzee (number 590) not protected from homologous HCV challenge after vaccination with E1 and E2 envelope proteins (*6*). As a control, chimpanzee infectious plasma was preincubated with preimmunization sera. The plasma was then incubated with beads that were coated with human TRX-EC2 (10 μg/ml), and HCV binding was assessed as described in (C). Results similar to that of serum from chimp 559 were obtained with sera from three other chimpanzees (numbers 357, 534, and 653) that were vaccinated and protected (*6*, *16*).

on B cells it associates with CD21 and CD19 (*19*), forming a complex that, when appropriately engaged, can lower B cell activation threshold (*20*). EBV and HCV target this complex by binding CD21 (*21*) and CD81, respectively. The B cell activation capacity of EBV is well known, and we have evidence that the HCV envelope protein E2 delivers a costimulatory signal to B cells (*22*). It may well be that the binding of HCV to CD81 on B lymphocytes in vivo lowers the activation threshold of these cells, thus facilitating the production of autoantibodies that are the hallmark of HCV-associated cryoglobulinemia (*4*).

Identification of the interaction between CD81 and HCV could help to elucidate the pathogenesis of HCV-associated diseases, obtain a small animal model of infection, and develop new therapeutic strategies directed at interfering with virus binding.

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7. Human cells (B and T lymphoma and hepatocarcinoma cell lines) were incubated with recombinant E2 expressed in mammalian cells (CHO) (*6*) and stained with biotin-labeled anti-E2 as described (*6*). Cells with the highest E2-binding capacity were sorted with a FacsVantage (Becton-Dickinson) and subcloned by limiting dilution. Growing clones were screened for E2 binding at the Facs, and clones with the highest mean fluorescence intensity were further expended.
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11. Fixed and embedded liver biopsy samples were used for immunohistochemical analyses. Briefly, air-dried, acetone-fixed cryostat sections were incubated with biotin-labeled recombinant E2 followed by incubation with peroxidase-labeled streptavidin.
12. For the construction of the thioredoxin-EC2 fusion (TRX-EC2), the human EC2 coding sequence was amplified from plasmid pCDM8-CD81 with the primers For-h.EC2 5′-GGCGGGGGTGGATCCGGGGGTGGAGGCTCGAGCTTTGTCAACAAGGACC-3′ and Rev-h.EC2 5′-CCCCAAGCTTTCACAGCTTCCCGGAGAAGAGGTCATCG-3′. The amplified DNA was digested with Xho I and Hind III and ligated to plasmid pThio-HisC (Invitrogen) digested with the same restriction enzymes. To clone the mouse EC2 sequence, we isolated RNA from mouse blood (RNeasy Blood Mini Kit; Qiagen). The primers For-mEC2 5′-AGGTCACTCGAGCTTCGTAAACAAAGACCAGATCG-3′ and Rev-mEC2 5′-AACTAACTGCAGCCCAAGCTTTCACAGGTTCCCAGAGAAGAGC-3′ were used for RT-PCR (Ready to go kit; Pharmacia). The RT-PCR product was digested with Hind III, filled in with Klenow enzyme, digested with Xho I, and cloned into plasmid pThio-HisC digested with Xho I and Stu I. The ligation mixtures (from human and mouse EC2) were used to transform *E. coli* Top10 competent cells, and one clone expressing the chimeric protein was used for further studies. For TRX-EC2 purification, *E. coli* Top10 (pThio-HisC-CD81) cells from a culture (500 ml) induced for 3.5 hours with 0.5 mM isopropyl-β-D-thiogalactopyranoside were treated with an ice-cold hypertonic solution [20 mM tris-HCl, 20% sucrose, and 2.5 mM EDTA (pH 8.0)] and subsequently with an ice-cold hypotonic solution [20 mM tris-HCl and 2.5 mM EDTA (pH 8.0)]. The proteins from the supernatant of the osmotically shocked cells were precipitated with 30% $(NH_4)_2SO_4$, resuspended in 15 ml of 20 mM phosphate buffer and 500 mM NaCl (pH 6.0), and loaded onto a 2-ml nickel-activated chelating Sepharose Fast Flow (Pharmacia) column. Retained proteins were eluted with a 30-mL 0 to 200 mM imidazole gradient, and the fractions containing the TRX-EC2 fusion were pooled, dialyzed against PBS, and stored at −80°C.
13. Purified TRX-EC2 recombinant proteins were subjected to SDS–polyacrylamide gel electrophoresis (SDS-

PAGE) in nonreducing conditions. After electroblotting, the PVDF membrane (Millipore) was probed overnight with recombinant E2 (1 μg/ml) at room temperature. Incubation with an mAb to E2 (clone 29.1A2) was followed by chemiluminescent detection with a peroxidase-conjugated polyclonal anti-mouse IgG (Amersham).

Polystyrene beads (1/4-inch diameter) (Pierce) were coated overnight with purified EC2 recombinant protein in citrate buffer (pH 4) at room temperature. After saturation for 1 hour with 2% bovine serum albumin in 50 mM tris-Cl (pH 8), 1 mM EDTA, and 100 mM NaCl (TEN) buffer, each bead was incubated at 37°C for 2 hours in 200 μl of TEN-diluted infectious chimp plasma containing 5×10^5 HCV RNA molecules. For inhibition experiments, the EC2-coated polystyrene beads were incubated with purified mAbs (50 μg/ml) for 1 hour at room temperature before incubation with the virus. Each bead was washed five times with 15 ml of TEN buffer in an automated washer (Abbot, Wiesbaden, Germany), and viral RNA was extracted with the Viral Extraction Kit (Qiagen). RNA (8 ml) was reverse transcribed at 42°C for 90 min in 20 ml of buffer A (Taq Man; Perkin-Elmer) containing 100 pmol of the HCV antisense primer CGGTTCCGCAGACCACTATG, 40 U of RNAsin (ribonuclease inhibitor) (Promega, Madison, WI), deoxynucleoside triphosphates (dNTPs) (250 μM), $MgCl_2$ (25 mM), and 10 U of Moloney murine leukemia virus reverse transcriptase (Boehringer). cDNA (20 μl) was amplified with a Perkin-Elmer ABI 7700 Sequence Detection System (45 cycles) in 50 μl of buffer A containing 100 pmol of the HCV sense primer TCTTCACGCAGAAAGCGTCTA, 5 pmol of the fluorescent detection probe 5′-(FAM)TGAGTGTCGTGCAGCCTCCAGGA(TAMRA) (FAM is carboxyfluorescein amino–modified oligo and TAMRA is tetramethylrhodamine amino–modified oligo), dNTPs (300 μM), and 1.25 U of Taq Gold (Perkin-Elmer). All reactions were quantified with HCV (genotype 1a)-infected plasma (branched DNA titer of 30 meq/ml) to generate a standard curve. Sequence Detector Software from Perkin-Elmer has been described previously (25). To evaluate virus binding to cells, we made human CD81+ mouse stable transfectants (NIH 3T3) that bound E2. However, we consistently failed to measure substantial virus attachment to the cell surface by PCR because of high background inherent to the technique.

15. In our assay, we captured only enveloped RNA molecules. The highest available concentration of human TRX-EC2 for coating beads was 100 μg/ml. At this concentration, about 7% of HCV input was bound by the beads. Using TRX-EC2 (100 μg/ml) and increasing numbers of beads, we captured about 10% of the HCV input, in terms of RNA molecules, further demonstrating that HCV binding is dependent on the CD81 concentration. Moreover, our experience with antibody (from mouse, chimp, or human)-coated beads has shown that the percentage of HCV that can be captured is negligible, further proving that CD81 is indeed a very effective binder of HCV particles.
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30. We thank C. Moretto for a contribution at the early stages of this project; S. Nuti and G. Salatti for help in flow cytometry; J. Kertsopon, D. Chien, Q.-L. Choo, S. Coates, K. Crawford, K. Berger, C. Dong, D. Piccioli, R. La Gaetana, and F. Mascioprinto for technical help and reagents; G. Corsi for artwork; and R. Rappuoli, G. Del Giudice, L. Gallì-Stampino, and N. Valiante for critical reading of the manuscript. We also thank D. Slade (Pharmacia and Upjohn) for providing the fluorescent detection probe.

22 June 1998; accepted 6 October

TNF-α Induction of CD44-Mediated Leukocyte Adhesion by Sulfation

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Regulation of cell adhesion is important for immune system function. CD44 is a tightly regulated cell adhesion molecule present on leukocytes and implicated in their attachment to endothelium during an inflammatory immune response. The proinflammatory cytokine tumor necrosis factor–α, but not interferon-γ, was found to convert CD44 from its inactive, nonbinding form to its active form by inducing the sulfation of CD44. This posttranslational modification was required for CD44-mediated binding to the extracellular matrix component hyaluronan and to vascular endothelial cells. Sulfation is thus a potential means of regulating CD44-mediated leukocyte adhesion at inflammatory sites.

During an immune response, activated leukocytes leave the circulation and enter the tissues. Leukocyte migration and extravasation is a multistep process involving the rolling and adhesion of leukocytes to the endothelium and their subsequent diapedesis to the inflammatory site (*1*). Activated T cells can bind the extracellular matrix component hyaluronan, and this CD44-mediated interaction has been implicated in the rolling and extravasation of lymphocytes at inflammatory sites (*2*). CD44 is normally present on leukocytes in an inactive state that cannot bind hyaluronan but can be converted to an active state upon appropriate stimulation, such as activation by antigen or cytokines (*3*, *4*). However, the molecular mechanism for this conversion is poorly understood. The binding ability of CD44 has been shown to be affected by three types of posttranslational modification: N- and O-linked glycosylation and glycosaminoglycan addition (*4*, *5*). Here, we show that sulfation is an additional posttranslational mechanism that can convert inactive CD44 to its active, adhesive form and that this mechanism is induced by the proinflammatory cytokine tumor necrosis factor–α (TNF-α).

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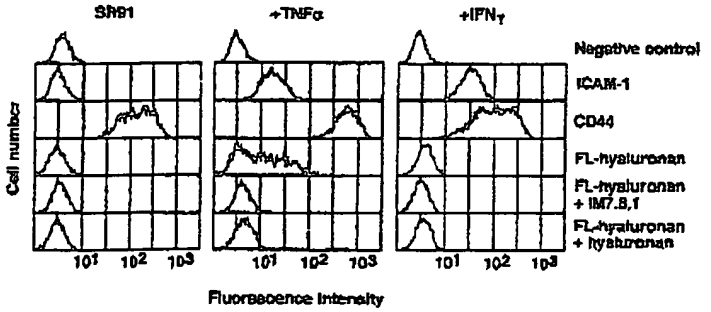

Fig. 1. Expression of CD44 and ICAM-1 and FL-hyaluronan binding ability on untreated, TNF-α–treated, or IFN-γ–treated SR91 cells by flow cytometry. Cells were preincubated with the CD44 mAb IM7.8.1, or with unlabeled hyaluronan, to block binding. Unlabeled SR91 cells were the negative control.